

Prenatal Exposure of Mice to Diethylstilbestrol Disrupts T-Cell Differentiation by Regulating Fas/Fas Ligand Expression through Estrogen Receptor Element and Nuclear Factor- κ B Motifs

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ABSTRACT

Prenatal exposure to diethylstilbestrol (DES) is known to cause altered immune functions and increased susceptibility to auto-immune disease in humans. In the current study, we investigated the effect of prenatal exposure to DES on thymocyte differentiation involving apoptotic pathways. Prenatal DES exposure caused thymic atrophy, apoptosis, and up-regulation of Fas and Fas ligand (FasL) expression in thymocytes. To examine the mechanism underlying DES-mediated regulation of Fas and FasL, we performed luciferase assays using T cells transfected with luciferase reporter constructs containing full-length Fas or FasL promoters. There was significant luciferase induction in the presence of Fas or FasL promoters after DES exposure. Further analysis demonstrated the presence of several *cis*-regulatory motifs on both Fas and FasL promoters. When DES-induced transcription factors were analyzed, estrogen receptor element (ERE), nuclear factor κ B (NF- κ B), nuclear factor

of activated T cells (NF-AT), and activator protein-1 motifs on the Fas promoter, as well as ERE, NF- κ B, and NF-AT motifs on the FasL promoter, showed binding affinity with the transcription factors. Electrophoretic mobility-shift assays were performed to verify the binding affinity of *cis*-regulatory motifs of Fas or FasL promoters with transcription factors. There was shift in mobility of probes (ERE or NF- κ B2) of both Fas and FasL in the presence of nuclear proteins from DES-treated cells, and the shift was specific to DES because these probes failed to shift their mobility in the presence of nuclear proteins from vehicle-treated cells. Together, the current study demonstrates that prenatal exposure to DES triggers significant alterations in apoptotic molecules expressed on thymocytes, which may affect T-cell differentiation and cause long-term effects on the immune functions.

Introduction

Diethylstilbestrol [DES; 4,4'-(3*E*)-hex-3-ene-3,4-diylidiphenol] is a potent synthetic estrogen. From the 1940s to the 1970s, pregnant women were administered DES to treat “pregnancy-associated” problems. Shortly after the link of prenatal DES exposure with adult onset of reproductive abnormalities was established, the use of DES was banned. However, by the time the use of DES in pregnant women was

banned, nearly 2 million to 5 million pregnant women had already been exposed to DES (Marselos and Tomatis, 1992a,b). Previous studies had demonstrated that prenatal exposure to DES can cause thymic atrophy and apoptosis in T cells (Brown et al., 2006a,b). There were also reports that neonatal exposure to DES had effects on the reproductive system (Herbst et al., 1971; Golden et al., 1998). However, the precise mechanism by which DES causes thymic atrophy or the fetal immune dysfunctions has not been thoroughly explored.

The murine thymus has been shown to be sensitive to DES when exposed for a short time during the neonate and adult stages of mice (Forsberg, 2000; Besteman et al., 2005; Brown et al., 2006a,b). Inasmuch as the thymus is the primary organ for the development of T cells that are long-lived and vital for immune competence, any alterations in this organ

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ABBREVIATIONS: DES, diethylstilbestrol; AP-1, activator protein-1; COMP, competition; DMSO, dimethyl sulfoxide; DRE, dioxin responsive element; EMSA, electrophoretic mobility-shift assay; ER, estrogen receptor; ERE, ER element; FasL, Fas ligand; GD, gestational day; Mut, mutant; NE, nuclear extract; NF-AT, nuclear factor of activated T cells; NF- κ B, nuclear factor κ B; PBS, phosphate-buffered saline; pCMV, porcine cytomegalovirus; PCR, polymerase chain reaction; PE, phycoerythrin; RLU, relative light units; RT, reverse transcription; SP1, specificity protein 1; TAM, tamoxifen; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VEh, vehicle.

may have notable immunological consequences. DES initiates early signaling primarily through the estrogen receptor (ER) and regulate the expression of various genes (Brown et al., 2006a,b; Miyagawa et al., 2011). Previous studies have demonstrated that DES caused a decrease in prothymocyte stem cells (Holladay et al., 1993) and a decrease in double positive CD4⁺CD8⁺ cells (Smith and Holladay, 1997; Brown et al., 2006a) and induced cell death in thymocyte subsets CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ (Calemine et al., 2002; Brown et al., 2006b). DES has also been shown to induce apoptosis of double negative CD4⁻CD8⁻ cells in a fetal thymic organ culture system (Lai et al., 2000). One of the key mechanisms of apoptosis is the extrinsic pathway involving Fas and FasL. Nair and Shaha (2003) have shown that DES caused apoptosis in spermatogenic cells of rat via Fas and the FasL pathway. In this study, therefore, we investigated the molecular mechanisms leading to the regulation of Fas and FasL by DES.

Regulation of a gene is controlled by distinct protein-DNA interactions at its promoter. Earlier studies had identified c-myc and Nur77 as transcription factors involved in the regulation of Fas and FasL expression (Davis and Lau, 1994). Several other transcription factors that include nuclear factor of activated T cells (NF-AT), p53, ceramide, members of c-Jun NH₂-terminal kinase/stress-activated protein pathways, and NF-κB have been identified to regulate the expression of Fas and FasL genes (Sharma et al., 2000). Studies from our laboratory have demonstrated the regulation of Fas and FasL involving DRE and/or NF-κB motifs present in their promoters (Singh et al., 2007). We have also shown that TCDD/aryl hydrocarbon receptor interaction in thymic stromal cells led to the activation of NF-κB and its migration from the cytosol to the nucleus, where it participated in up-regulation of FasL expression (Camacho et al., 2005). Furthermore, NF-κB has also been shown to up-regulate the expression of Fas (Puga et al., 2000; Schlezinger et al., 2000; Sulentic et al., 2000; Baba et al., 2001; Zheng et al., 2001).

The precise mechanism of DES-mediated regulation of Fas and FasL genes with respect to the specific transcription factors has not been well characterized and understood. Therefore, the aim of the present study was to elucidate the molecular mechanisms of DES-mediated regulation of Fas and FasL gene expression during the development of the immune system and examine the role of ERE and NF-κB motifs in the regulation of these genes.

Materials and Methods

Mice. Normal and pregnant (GD 14) mice (C57BL/6: H-2^b) were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were housed at the University of South Carolina Animal facility. Care and maintenance of the animals were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Cell Line. The mouse T cell lymphoma cell line (EL4) was maintained in complete RPMI 1640 medium (10% heat-inactivated fetal bovine serum, 10 mM L-glutamine, 10 mM HEPES, and 100 µg/ml penicillin/streptomycin) at 37°C and 5% CO₂.

Reagents and Antibodies. DES was purchased from Sigma-Aldrich (St. Louis, MO). HEPES, L-glutamine, RPMI 1640, PBS, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal antibodies [anti-mouse IgG, FcBlock, anti-FasL-PE (Kay-10), and anti-Fas-PE (Jo2)] were purchased from BD

Pharmingen (San Diego, CA). A TransFactor Universal Chemiluminescent kit was purchased from Clontech Laboratory, Inc. (Mountain View, CA). pGL3-basic vector was from Promega (Madison, WI); pCMV-β-galactosidase vector was from Clontech Laboratory, Inc.; an Amaxa Cell line Nucleofector transfection kit and Mouse T Nucleofector transfection kit were purchased from Lonza Cologne GMBH (Cologne, Germany); and the Dual-Light system was purchased from Applied Biosystems (Foster City, CA). A RNeasy Mini kit and iScript cDNA synthesis kit were purchased from QIAGEN (Valencia, CA). Epicenter's PCR premix F and Platinum Taq Polymerase kits were purchased from Invitrogen. T4 polynucleotide kinase was purchased from New England Biolabs (Ipswich, MA), and NICK columns were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Anti-Fas primary polyclonal antibody (Ab-1) was purchased from Calbiochem (San Diego, CA), and anti-FasL primary polyclonal antibody (AB16982) was purchased from Millipore Corporation (Billerica, MA).

In Vivo Exposure of Pregnant Mice with DES. To determine the effect of DES on the thymus in normal adult mice and their fetuses, a single dose of DES (5 µg/kg) was administered (intraperitoneally) into pregnant C57BL/6 mice on GD 14. We chose to use GD 14 pregnant mice for all of our experiments, because by that time fetuses are not fully developed, so the effects of DES can be fully exploited. In addition, we used DES (5 µg/kg) based on previous studies from our laboratory (Brown et al., 2006a) and studies from other laboratories (Yoshida et al., 2011; Rowas et al., 2012). On days 2 (GD 16) and 4 (GD 18) post-DES treatment, thymic weight and cellularity of both mothers and fetuses were determined. Mice treated with vehicle (VEH; corn oil) were used as control. For each treatment group, at least three pregnant mice were used, and from each pregnant mother we obtained an average of eight pups. To reduce the variability among the pups in each litter, we combined the three litters from each treatment group to generate a pool of ~24 pups. Because of low thymic cellularity in the fetus, thymi from approximately five pups were randomly pooled per sample, and the replicate pools were used for statistical analysis.

Preparation of Thymocytes and Detection of Apoptosis in Thymocytes. Thymi from mice and fetuses were harvested and placed in complete RPMI 1640 medium. Single-cell suspensions of thymi were prepared as described previously (Camacho et al., 2004a,b). Cell viability was determined on a hemacytometer (Hausser Scientific, Horsham, PA) by staining the cells with trypan blue dye and using an inverted phase-contrast microscope (Nikon, Inc., Melville, NY). For calculating thymic cellularity, the data were expressed as total number of thymocytes/mice. For statistical analysis, five to six replicate pools were compared from each treatment group and depicted as mean ± S.E.M.

Thymocytes from mice exposed to DES or VEH were analyzed for apoptosis by using the TUNEL assay kit (Roche Diagnostics, Indianapolis, IN) as described previously (Camacho et al., 2004a,b, 2005; Singh et al., 2008). In brief, thymocytes (1 × 10⁶) from various groups of mice were cultured for 24 h in complete RPMI 1640 medium. The next day, the cells were washed twice with PBS and analyzed by TUNEL assay. Apoptotic cells were quantified as the average number of TUNEL-positive cells from at least 30 foci of each section.

Determination of Fas and FasL Expression in Fetal Thymic Cells. Expression of Fas and FasL in fetal thymic cells was determined by performing RT-PCR. In brief, single-cell suspension of fetal thymic cells after VEH or DES exposure was prepared as described above. Expression of Fas and FasL was determined by RT-PCR as described previously (Singh et al., 2007) and Western blotting. For RT-PCR, total RNAs from the VEH- or DES-treated groups were isolated by using the RNeasy Mini Kit and following the protocol of the company (QIAGEN). First-strand cDNA synthesis was performed in a 20-µl reaction mix containing 2 µg of total RNA using the iScript Kit and following the protocol of the manufacturer (Bio-Rad Laboratories). PCR was performed by using mouse FasL- or Fas-specific sets of forward and reverse primers as described previously

(Singh et al., 2008). The PCR products generated from mouse Fas and FasL primer pairs were normalized against PCR products generated from mouse 18S-specific forward 5'-GCCCGAGCCGCTG-GATAC-3' and reverse 5'-CCGGCGGGTCATGGGAATAAC-3' primers after electrophoresis on 1.5% agarose gel and visualization with UV light. The band intensity of PCR products was determined by using the Bio-Rad Laboratories image analysis system. Expression of Fas and FasL in thymi was also confirmed by performing Western blotting using anti-Fas primary polyclonal antibody (Ab-1; Calbiochem) and anti-FasL primary polyclonal antibody (AB16982; Millipore Corporation).

Determination of Fas and FasL Expression in Unactivated and Activated T Cells. Expression of Fas and FasL in unactivated and activated T cells was analyzed by using flow cytometry and RT-PCR. In brief, unactivated purified T cells or ConA (2 μ g/ml overnight)-activated T cells from C57BL/6 mice were cultured in the presence of VEH or DES (10 μ M), tamoxifen (TAM) (10 μ M), or TAM+DES (10 μ M) for 8 h. We chose to use 10 μ M DES or TAM based on previously established studies (Lai et al., 2000), including those from our laboratory (Brown et al., 2006a,b), where various doses (1–20 μ M) of DES were used. We found that 10 μ M DES or TAM was the optimal concentration for examining the molecular mechanisms. Expression of Fas and FasL was determined by flow cytometry or RT-PCR as described previously (Singh et al., 2007). For flow cytometry, cells were stained with fluorescein isothiocyanate-labeled anti-mouse Fas and PE-labeled anti-mouse FasL antibodies, and their expression was analyzed by flow cytometry (FC500; Beckman Coulter, Fullerton, CA). For RT-PCR, total RNAs from various treated groups were isolated by using the RNeasy Mini Kit and following the protocol of the manufacturer (QIAGEN). First-strand cDNA synthesis and PCR was performed by using mouse FasL- or Fas-specific sets of forward and reverse primers as described previously (Singh et al., 2008). The PCR products, generated from mouse Fas and FasL primer pairs, were normalized against PCR products generated from mouse 18S after electrophoresis on 1.5% agarose gel and visualization with UV light. The band intensity of PCR products was determined by using the Bio-Rad Laboratories image analysis system.

Generation of the Mouse Fas and FasL Promoter Reporter Constructs. The mouse Fas or FasL promoter was cloned in pGL3 vector and was described previously (Singh et al., 2007). To elucidate the role of various *cis*-regulatory motifs present in the Fas or FasL promoter involved in the regulation of Fas or FasL expression, pGL3-Fas-Luc or pGL3-FasL-Luc reporter constructs were used.

Transfection of EL4 Cells and Luciferase Assays. Freshly cultured EL4 cells (5×10^6) were transfected with 5 to 10 μ g of the indicated reporter plasmid and 1 μ g of the pCMV- β -galactosidase control vector by using an Amaxa Nucleojector instrument and kits from Lonza Cologne GMBH following the protocol of the company. Two days after transfection, EL4 cells were replated in triplicate in 96-well plates, and the cells were treated with VEH or various doses of DES (1, 5, 10, and 20 μ M/ml) and incubated for 24 h at 37°C, 5% CO₂. After incubation, cells were harvested, washed with PBS, and centrifuged, and extracts were prepared by lysing cells in 70 μ l of reporter lysis buffer (Promega). Luciferase activity was determined by mixing 20 μ l of extract with 100 μ l of luciferase substrate (Promega) and immediately reading the sample in Victor² (PerkinElmer Life and Analytical Sciences, Waltham, MA). Likewise, β -galactosidase activity was determined by mixing 20 μ l of extract with galactolite β -galactosidase substrate (Tropix, Bedford, MA), incubating it for 1 h at 25°C, adding 300 μ l of galactolite accelerator (Tropix), and reading the sample with Victor². Under similar conditions, TAM (10 μ M/ml), an antagonist for DES, was used in the culture at least 1 h before DES treatment, and luciferase and β -galactosidase assays were performed. Luciferase activity was normalized by dividing the mean luciferase RLU by the mean β -galactosidase RLU, and the normalized luciferase RLU from the stimulated samples were di-

vided by the normalized RLU of the untreated sample, and values were expressed as normalized-fold induction.

Identification of Transcription Factor Binding Motifs in Fas and FasL Promoters. Each promoter possesses several transcription binding motifs with which transcription factors bind to regulate gene expression. To identify various transcription factors binding motifs present in the Fas and FasL promoters, we analyzed the Fas or FasL promoters by using MatInspector 8.0 software from Genomatix (Munich, Germany). This software helps identify various transcription factor binding sites present in a promoter.

Generation of Transcription Factor Binding Site-Specific Probes for Fas and FasL Promoters. Probes for various transcription factors binding sites present in Fas or FasL promoters were generated. At the 5' end, the probes were biotinylated. Probes and complementary strand were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequence of various probes containing transcription factors binding sites present in Fas or FasL promoters were as follows. Underlining in the probes shows nucleotides for the respective motifs in the probes.

Probes for the Fas promoter were: ERE, 5'-CTCCCCCACTT-GATCTTCCCATCATACTACC-3', 3'-GAGGGGGTGAAGACTAG-AAGGGTAGTATGATGG-5'; NF- κ B1, 5'-ATTGTTGGGGACTC-CCATGCAAC-3', 3'-TAACAAACCCCTGAGGGTACGTTG-5'; NF- κ B2, 5'-GAAGCCAGGGACATTCCCTCATCAGGA-3', 3'-CTTCGGTCCCTGTAAGGGGAGTAGTCCT-5'; NF- κ B3, 5'-GCTCTGTGGAGGGACCTCCCTCATCAGAAT-3', 3'-CGAGACACCTCCCTGGAGGGAGTAGTCTTA-5'; NF- κ B4, 5'-CGC-CCAGGATGGGAATGCCCATTTATGCAAT-3', 3'-GCGGGTCTACCCCTTACGGGTAAATACGTTA-5'; NF- κ B5, 5'-GTGAGT-CAGTGGGTTCCTCCCGGGAGACCCAGCA-3', 3'-CACTGAGTC-ACCCAAAGGGGCCCTCTGGTCTGT-5'; NF-AT1, 5'-CCCCATC-CAGGAAAGGAAACCTTCAT-3', 3'-GGGGTAGGTCTCTTTC-CTTTTGAAGTA-5'; NF-AT2, 5'-ACTCTGCTAGGTTTCCATAC-CTCTCAAATG-3', 3'-TGAGACGATCCAAAGGTATGGAGAGT-TTAC-5'; AP-1.1, 5'-CTGTGTTATTTGTCATAGTCATTACCT-TATTC-3', 3'-GACACAATAAACAGTATCAGTAAATGGAATAAG-5'; AP-1.2, 5'-GCAGACCTGTCTCATGACAAAATATTGAAAGT-3', 3'-CGTCTGGACAGTGAAGTGTTTTATAACTTTCA-5'; AP-1.3, 5'-GACCTGAGGGTGTGACTGTGGAAGCGCCCA-3', 3'-CTG-GACTCCACACTGACACCTTCGCGGGT-5'; and DRE and SP1, 5'-GGTTTGTGCGTGCCAGGGGGCGGGCCTAGG-3', 3'-CCAAA-CACGCACGGTCCCCCGCCCGGATCC-5'.

Probes for the FasL promoter were: ERE, 5'-TTTTCATCTGGT-GACCAGAAGAGAGATTGC-3', 3'-AAAAGTAGACCACTGGT-TCTTCTCTCTAACG-5'; NF- κ B1, 5'-CTCCTTGCTCTTTTC-CCCATGCCTCAGCA-3', 3'-GAGGAACCAGAAAAGGGGTACG-GAGTCGT-5'; NF- κ B2, 5'-ACAGGAGAAAGGTGTTTCCCTT-GACTGC-3', 3'-TGCTCTCTTTCCACAAAGGGAAGTACG-5'; SP1 and NF-AT1, 5'-TCAGAAATTTCTGGGCGGAAACCTT-CTGGGG-3', 3'-AGTCTTTAAAGACCCGCTTTGAAGGACCCC-5'; NF-AT2, 5'-CCTTGACTGCGGAAACCTTATAAAACT-3', 3'-GGAAGTACGCTTTTGAATATTTTGA-5'; and NF-AT3, 5'-AAGGCTGTGAAGAGGAAACCTTTTCTCTGG-3', 3'-TTCCGA-CACTTCTCCTTTGGGAAAGGACC-5'.

Identification of Transcription Factors after DES Treatment of T Cells. After identification of various transcription factor binding sites present in the Fas and FasL promoters, we sought to identify transcription factors involved in the DES-mediated regulation of Fas and FasL genes. To this end, we performed in vitro and chemiluminescent assays to identify various transcription factors involved in the DES-mediated regulation of Fas and FasL expression in T cells. In brief, we first prepared nuclear extracts (NEs) from T cells treated with VEH or DES (10 μ M/ml) for 6 h by using the TranFactor extraction kit from Clontech Laboratory, Inc. following the protocol of the company. Next, we performed chemiluminescent assays using the TranFactor Universal Chemiluminescent kit from Clontech Laboratory, Inc. following the protocol of the company. The

binding of transcription factors with various Fas or FasL probes were identified by Victor² (PerkinElmer Life and Analytical Sciences).

Electrophoretic Mobility-Shift Assay. The EMSAs were performed using oligonucleotide probes corresponding to ERE and NF- κ B motifs of the Fas or FasL promoters and nuclear proteins prepared from T cells treated with VEH or DES.

Preparation of Nuclear Extracts. To prepare nuclear extract for EMSAs, purified T cells from C57BL/6 mice were treated with DES (10 μ M/ml) and cultured for 6 h at 37°C, 5% CO₂. After DES or VEH treatments, nuclear extracts from various treated groups were isolated as described previously (Singh et al., 2007). The protein concentration was determined by using the bicinchoninic acid protein determination kit from Thermo Fisher Scientific (Waltham, MA), using albumin as a protein standard. The supernatant containing nuclear extract was snap-frozen in liquid nitrogen and stored at -80°C.

Generation of Oligonucleotide Probes. To perform EMSAs, hairpin oligonucleotide probes corresponding to ERE and NF- κ B motifs of mouse Fas or FasL promoters were synthesized at Integrated DNA Technologies. The sequences of various oligonucleotide probes for Fas or FasL promoter are as follows. Underlining in the probes shows nucleotides for the respective motifs in the probes.

Mouse Fas promoters were: hairpin loop probe for Fas ERE motif, GAGAAACTTGATCTTCCCATTTTAGCTAAAATGGGAAGTCAAGTTTCT; hairpin loop probe for Fas ERE mutant motif, GAGAAAACCCTATAGGACAATTTTAGCTATGGAAAATTGTCCTATAGGGTTTCT; hairpin loop probe for Fas NF- κ B1 motif, GAGAAAAGGGGACTCCCTTTAGCTAAAAGGGAGTCCCTTTTCT; and hairpin loop probe for Fas NF- κ B2 motif, GAGAAAAGGGACATTC-CCCTTTAGCTAAGGGGAATGTCCCTTTTCT.

Mouse FasL promoters were: hairpin loop probe for ERE motif, GAGAAAATGGTGACCAGTTTAGCTTAAACTGGTCACCATTTTCT; hairpin loop probe for ERE mutant motif, GAGAAAATGGCTGTTAGTTTACTTAAACTAACAGCCATTTTCT; hairpin loop probe for NF- κ B1 motif, GAGATGGTCTTTTCCCCAATTAGCTTTTGGGGAAAAGACCATCT; hairpin loop probe for NF- κ B1 mutant motif, GAGATGGTCTTTTAAACAATTAGCTTTTGTTTAAAAGACCATCT; hairpin loop probe for NF- κ B2 motif, GAGAAAAGGTGTTTCCCTTTTAGCTTAAGGGAAACACCTTTTCT; and hairpin loop probe for NF- κ B2 mutant motif, GAGAAAAGGTGTTTAAATTTAGCTTAATTTAAACACCTTTTCT.

The hairpin oligonucleotide probes were 5-end-labeled by mixing 1 pmol of hairpin oligonucleotide with 10 μ Ci of [γ -³²P]ATP (MP Biochemicals, Aurora, OH), and eight units of T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA) in 1 \times PNK buffer and incubating them for 1 h at 37°C. After incubation, the end-labeled hairpin oligonucleotides were purified from free ATP by passing over a NICK column (GE Healthcare). One to 5 μ g of nuclear protein was mixed with 1 μ l of radiolabeled oligonucleotide (40,000 cpm) in a reaction mix containing 1 μ l of binding buffer [10 mM Tris, 1 mM

EDTA, 1 mM dithiothreitol, 100 mM KCl, 10% (v/v) glycerol] and 1 μ g of poly(dI-dC) (GE Healthcare) as a nonspecific inhibitor in a final volume of 25 μ l and incubated for 30 min at 25°C. The samples were resolved on a 6% polyacrylamide gel in Tris borate EDTA that had been prerun for 30 min. The gels were dried and exposed to X-ray film. For the specific and nonspecific competition analyses, equimolar amounts of the cold hairpin oligonucleotide competitors were added to the binding reaction before the addition of the labeled oligonucleotide probes.

Statistical Analysis. Data presented for luciferase expression were derived from at least three independent experiments and are depicted as mean \pm S.E.M. Results for RT-PCR represent at least three animals for each treatment and are expressed as the mean \pm S.E.M. Statistical analyses were performed by using Student's *t* test

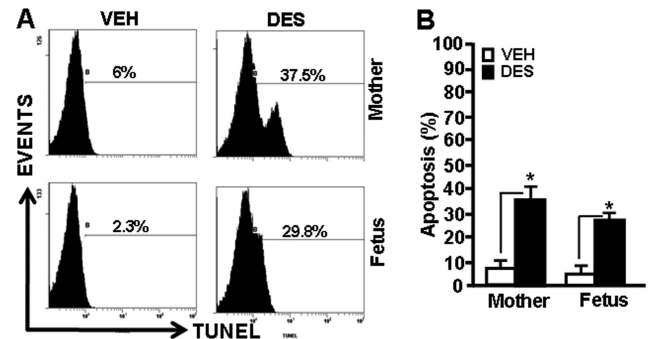


Fig. 2. DES triggers apoptosis in the thymocytes of pregnant mothers and fetuses. The thymocytes from mothers and fetuses on day 4 (GD 18) after DES treatment were analyzed for apoptosis by using TUNEL assay. A, a representative histogram of three independent experiments, B, data from groups of five mice (mean \pm S.E.M.). *, statistically significant ($p < 0.05$) difference in apoptosis between DES- and VEH-treated groups.

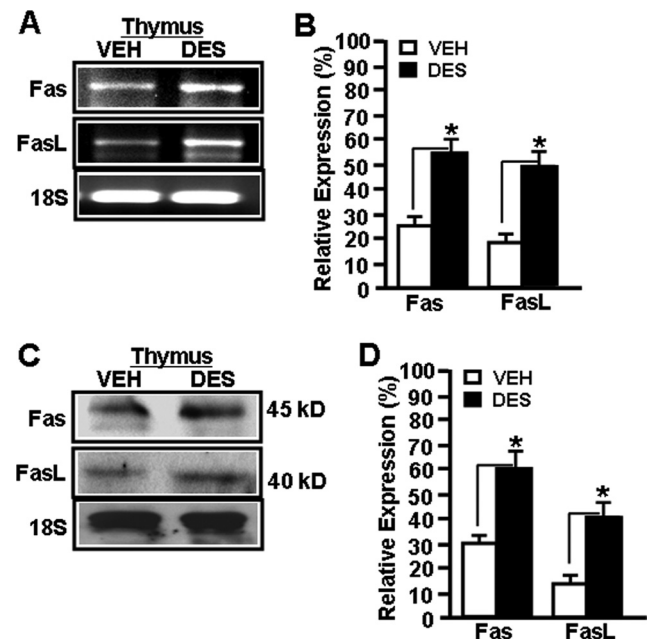


Fig. 3. Expression of Fas and FasL in fetal thymic cells after prenatal exposure to DES. A and B, expression of Fas and FasL in fetal thymic cells was determined by RT-PCR 24 h after DES or VEH treatments. Data in A are representative of three independent assays and are plotted in B as mean \pm S.E.M. *, statistically significant ($p < 0.05$) difference in the expression of Fas and FasL between VEH- and DES-treated thymic cells. C and D, expression of Fas and FasL in fetal thymic cells was determined by Western blot. Data in C are representative of three independent experiments and are plotted in D as mean \pm S.E.M. *, statistically significant ($p < 0.05$) difference in the expression of Fas and FasL between VEH- and DES-treated thymic cells.

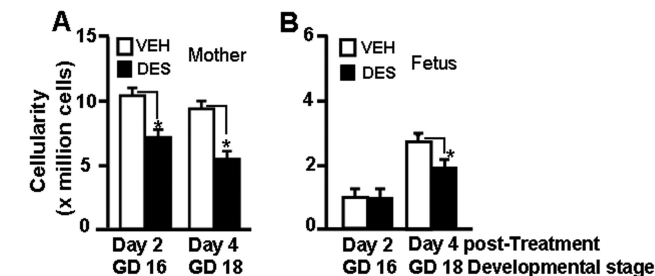


Fig. 1. Effect of DES on thymic cellularity of pregnant mice (A) and fetuses (B). Pregnant mice (GD 14) were injected intraperitoneally with VEH (control) or DES (5 μ g/kg body weight). On days 2 (GD 16) and 4 (GD 18) after DES or VEH treatment the thymi from mothers and their fetuses were harvested to determine thymic cellularity. Data from groups of five mice are depicted as mean \pm S.E.M. Vertical bars represent mean \pm S.E.M. *, statistically significant ($p < 0.05$) differences between the indicated groups.

or two-factor analysis of variance as appropriate, with a p value of ≤ 0.05 considered to be statistically significant.

Results

DES Induces Alterations in Thymic Cellularity in Pregnant mice and Their Fetuses. In the current study, we determined DES-induced changes in thymic cellularity in pregnant mothers and their fetuses. To this end, a single dose of DES (5 μ g/kg) was administered (intraperitoneally) into pregnant C57BL/6 mice on GD 14. On days 2 (GD 16) and 4 (GD 18) after DES treatment, thymic cellularity of both mothers and fetuses was determined. We had shown previously that pregnant mice exhibit a dramatic decrease in thymic cellularity compared with nonpregnant mice, and exposure to environmental contaminants, such as TCDD, further decreases their numbers (Camacho et al., 2004a). We

noted that, unlike nonpregnant mice that showed ~ 250 million thymocytes/mouse (Camacho et al., 2004a), pregnant mice on GD 16 had fewer than ~ 11 million cells/mouse. This cellularity was further decreased after treatment with DES (Fig. 1A). When we pursued similar studies in the fetus, DES was found to alter thymic cellularity in the fetus on GD 18 but not on GD 16 (Fig. 1B). These data demonstrated that exposure to DES during pregnancy decreases thymic cellularity in both the mothers and fetuses.

Detection of DES-Induced Apoptosis in Thymocytes of Pregnant Mice and Their Fetuses. Thymi from VEH- or DES-exposed pregnant mice and fetuses were evaluated for apoptosis as described previously (Brown et al., 2006a,b; Singh et al., 2011). The results from a representative experiment are shown in Fig. 2A, and data from multiple experiments are plotted in Fig. 2B. The thymocytes from DES-

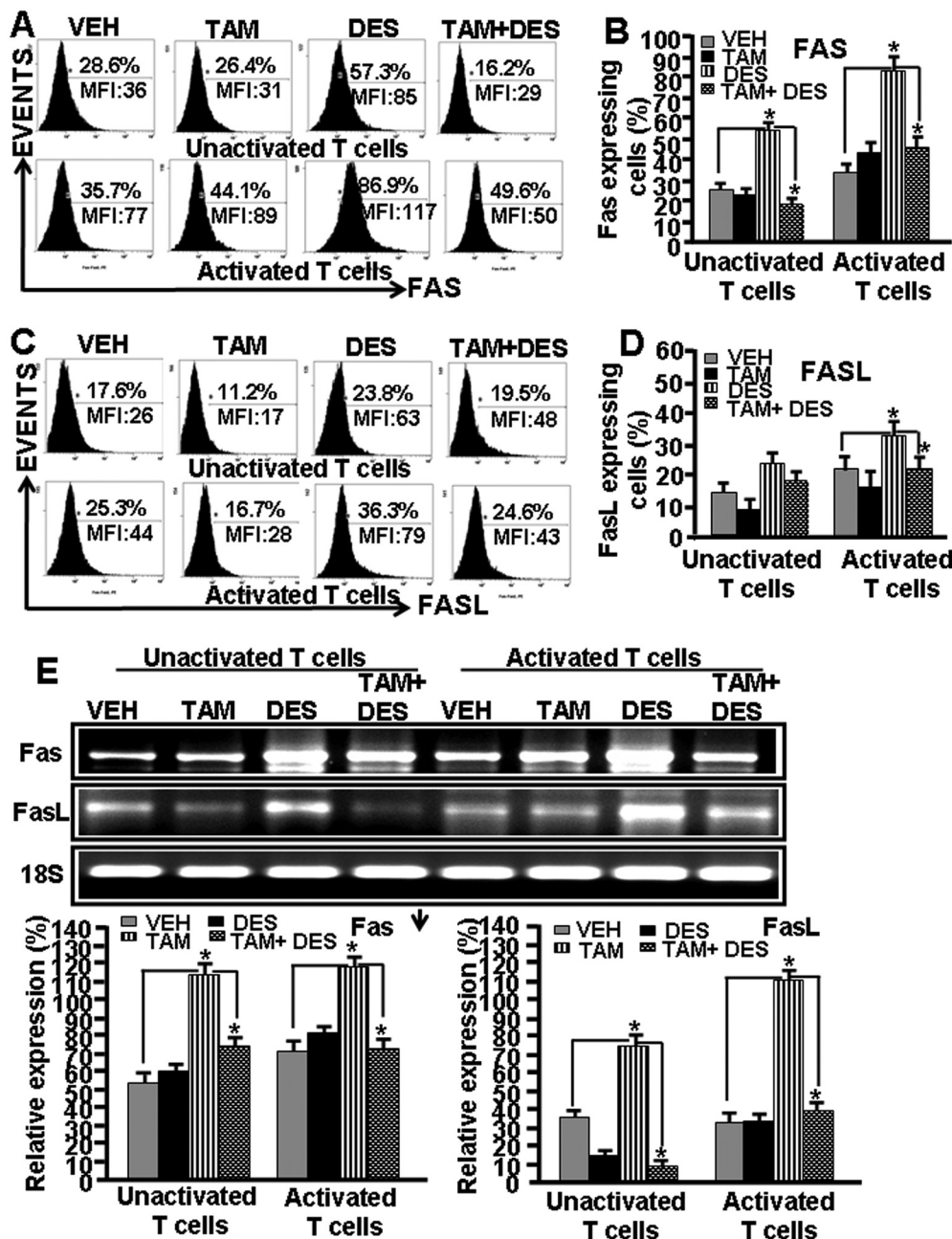


Fig. 4. Expression of Fas and FasL in T cells in the presence of DES. Unactivated or ConA-activated T cells were cultured in the presence of VEH (DMSO) or DES (10 μ M) for 24 h. Expression of Fas and FasL in unactivated and activated T cells was determined by flow cytometry (A–D) or RT-PCR (E) 24 h after DES treatments. Data in A and C are representative of three independent assays and are plotted in B and D, respectively as mean \pm S.E.M. *, statistically significant ($p < 0.05$) difference in the expression of Fas and FasL between VEH- and DES-treated groups. Data in E represent semiquantitative RT-PCR and are mean \pm S.E.M. of three independent experiments. Expressions of Fas and FasL are presented as percentage of 18S expression in the y-axis, and expression of 18S was considered to be 100% for each experiment. Fas and FasL expression was noted to be significantly ($p < 0.05$) increased. *, statistically significant ($p < 0.05$) difference in the expression of Fas and FasL between VEH- and DES-treated groups. TAM, an antagonist of ER, was also tested.

treated groups showed a significantly higher percentage of apoptosis in both the mothers and fetuses GD18 (Fig. 2) compared with the VEH-treated control groups.

DES Up-Regulates Fas and FasL Expression in Fetal Thymic Cells. Next, we determined the expression of Fas and FasL, key molecules involved in apoptosis, in fetal thymic cells exposed to VEH or DES on GD 18 by performing RT-PCR. Thymic cells from both groups showed low-level expression of Fas and FasL in the presence of VEH (Fig. 3, A and B). However, there was significant up-regulation of both Fas and FasL expression in mice prenatally exposed to DES (Fig. 3, A and B). Data obtained from Western blot analysis confirmed up-regulation of both Fas and FasL expression in fetal thymic cells after DES exposure (Fig. 3, C and D). These data demonstrated that DES induces expression of both Fas and FasL in fetal thymic cells.

DES Up-Regulates Fas and FasL Expression in T Cells via the ER. Next, we investigated whether the up-regulation of Fas and FasL by DES was mediated through the activation of the ER. To this end, unactivated or ConA-activated T cells from normal C57BL/6 mice were cultured in the presence of VEH (DMSO) or DES (10 μ M/ml) in the presence of TAM (10 μ M/ml) or VEH. T cells were either analyzed by flow cytometry or RT-PCR after treatments as described previously (Singh et al., 2007). More than 25% of all unactivated T cells and more than 35% of activated T cells expressed Fas (Fig. 4, A and B) in the presence of VEH, whereas both unactivated and activated T cells upon treatment with DES expressed significantly higher levels of Fas as indicated by increases in mean fluorescent intensity and Fas-expressing cells (Fig. 4, A and B). These data demonstrated that DES up-regulates Fas expression in both unactivated and activated T cells. In the presence of TAM (an antagonist of ER), DES failed to increase the expression of Fas in both unactivated and activated T cells, demonstrating that DES regulates Fas expression via the ER (Fig. 4, A and B). Furthermore, unactivated or activated T cells upon treatment with TAM alone did not show significant change in the expression of Fas compared with VEH-treated T cells (Fig. 4, A and B). Similar to Fas expression, DES significantly increased the percentage of FasL-expressing cells in both unactivated and activated T-cell cultures (Fig. 4, C and D). DES in the presence of TAM did not affect FasL expression, demonstrating that TAM blocked DES-ER-induced up-regulation of FasL expression. The presence of TAM alone did not effect FasL expression in both unactivated and activated T cells (Fig. 4, C and D). RT-PCR data further corroborated the results generated by flow cytometry and confirmed that DES caused up-regulation of both Fas and FasL in unactivated and activated T cells (Fig. 4E).

DES Regulates Fas and FasL Expression Through Their Promoters. To determine DES-mediated regulation of Fas and FasL expression through their promoters, EL4 T cells were transfected with constructs containing the full-length upstream region of Fas (pGL3-Fas-Luc) or FasL (pGL3-FasL-Luc) promoter. Two days post-transfection, the transiently expressing EL4 cells were treated with various doses of DES (1–20 μ M) or VEH (DMSO). Luciferase assays were performed 24 h after DES or VEH treatment. We observed a DES-mediated increase in the expression of luciferase when Fas or FasL promoter was used for transfection and the cells were treated with DES for at least 24 h (Fig. 5A). The effect of DES on luciferase expression was very

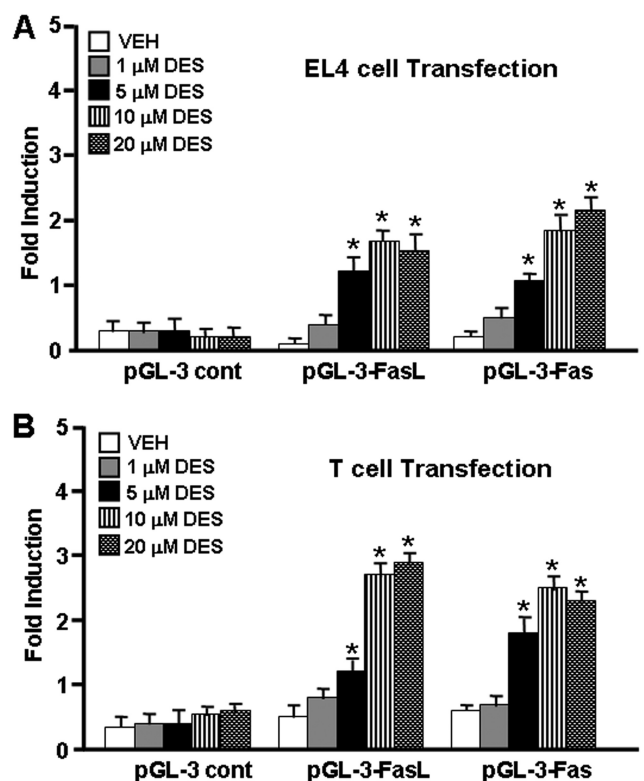


Fig. 5. DES regulates Fas and FasL promoter activity. EL4 cells (A) or T cells (B) were transfected with luciferase reporter constructs containing full-length Fas promoter (pGL3-Fas) or FasL (pGL3-FasL) or pGL3 control and pcDNA3.1 containing pCMV- β -galactosidase gene. Two days post-transfection, the transiently expressing EL4 or T cells were treated with various doses of DES (1, 5, 10, and 20 μ M/ml) or DMSO (VEH). The luciferase activity was normalized to β -galactosidase activity and expressed as normalized fold induction. The vertical bars represent mean \pm S.E.M. from three independent experiments. *, statistically significant ($p < 0.05$) difference in luciferase expression between VEH and DES-treated groups.

minimal at lower doses (1 μ M; Fig. 5A). At 5 μ M the effect of DES was moderately altered. However, we observed a highly significant increase ($2-3 \pm 0.5$ -fold) in luciferase expression when 20 μ M DES was used (Fig. 5A). We observed minimal levels of luciferase expression when EL4 cells were transfected with Fas or FasL promoter and treated with VEH as well as in pGL-3 controls (Fig. 5A). The observed up-regulation of luciferase expression was Fas or FasL promoter-specific and DES-induced, because the luciferase expression was significantly higher ($p < 0.05$) in cells transfected with Fas or FasL promoter compared with untreated or VEH-treated cells (Fig. 5A). Similar results were obtained in T cells transfected with Fas (pGL3-Fas-Luc) or FasL (pGL3-FasL-Luc) promoter in the presence of DES (Fig. 5B). The data obtained demonstrated that DES-induced up-regulation of luciferase expression was through the Fas or FasL promoter.

Identification of Various Transcription Factor Binding Motifs in Mouse Fas and FasL Promoters. To understand DES-induced regulation of Fas and FasL expression, we first identified various transcription factor binding motifs in Fas and FasL promoters by using MatInspector 8.0 software from Genomatrix as described under *Materials and Methods*. We identified the following transcription factor binding motifs in mouse Fas promoter: one ERE, five NF- κ B (NF- κ B1, NF- κ B2, NF- κ B3, NF- κ B4, and NF- κ B5), three

A Identification of various regulatory elements in Fas promoter

-1272 TGTTGGCATGTAGGGATGAAGGCTCTATGTAGGCACTTTCTTCTGCTCTCAAC
TTCTCTATATTCATGAGTTGTGTGGGTCTCAGTTTGTGGGGAGCATCTATAGTCTTGGC
AACAGGCTGCATTGTTTGGGGACTCCCATGCAACCTTTTGGCCAACGATTCAATTAGAT
GCAACCCCATCCAAGAAAGGAACTTCATTTGATGACAAGAGATGTTCACTTGTGGCT
CTGTCTCCCCCATTTATTTGGGTATAGATCACCTTCATGTATGCTTATACCTTTAGGAAGAC
TCTGCTAGGTTTCCATACCTCTCAAAATGCCCTTTAATTTTAGTGTCTCTCCCTGTATTC
CCATTTCATCGTCTTCTTCCCTTCCCTCCCTCCCTCCCTAGTCCCTTACTCTATATCTAA
ATTCCTCTCTTAATAAGATCTTTCTGTCCCTCCCTAGTCCCTTACTCTATATCTAA
CCCCCGTGGTTCTAAAGATTCTGGCTTGGTCCCTGAGTTAACTGCTGTGTTATTTGTCAT
AGTCATTTACCTTATTCTCCATTTGGGAAACGAAGCCAGGGACATTCCCTCATCAGGAT
CCCAAAGCCTATTATCTCTACCTCTGCTGTGGTGATTGTCACCTTCATGTATCATATGA
GCTCTGTGGAGGGACCTCCCTCATCAGAACTCCTAGGGTGTGACCTCCTCTGTGGTAATT
ACCATTCAGAGGCCCCAGAGCCCGCAGCAGACCTGTCTACTGACAAATATTGAAAGTGA
AGGCAAAATGGTCTTTGTCTCCACAATCAGCCAAAGTCCAAATCAATACAGAGTTCAGAA
TTCTTCCAGGGTGAGCAGCAGGACAGAAAAAATCTCACTTGACCTGAGGGGTGTGAC
TGTGGAAGCGCCAGGATGGGGAATGCCATTTATGCAATCAAGCCCTGCTTGGCCCCC
TCCTCCCCCGCCCCACCCCGCTCAGGCCGCTGTGCAAGTGGTGAGTCAGTG
GGTTTCCCGGGAGACCAGCAGAACTTTCTGCACCCTTGGGGCTTTACGAAGATTGTG
TTTGGTCCCTCAAAGAAAAAAGTGACATACAGTGTTCAAAGCGATTCTTGGGAAGA
CTGAATAGAGCGAAGCGGTTTGTGCGTGCCAGGGGGCGGCCTAGGCCAAAGTACAT
ACCCACAGGCAGTCTAGAGCTGGTGGGTGGGCCGAGGCTGCCACACAGGCCGCC
GCTGTTTCCCTTGTGTCAGACATG

Red: ERE,

Black: NF- κ B1 to NF- κ B5,

Violet: AP1,

Blue: SP1,

Orange: DRE,

Green: NF-AT

B Identification of various regulatory elements in FasL promoter

-786GTACCTCAGTTTTCATCTGGTGACCAGAAAGAGAGATTGCAAGTTAAATGATTACA
GAGGAGGAAGCTACATTTCCAGGGGAAGTGGGATGGATAGGGGGCAGGCACAGTG
GGGGGTTAGGGCAGCCTTGAACACCTGGCAGACATTCTGTTGACAGTGGCTGATGT
TCAGGGAAGGGACTTCAACAGAGGATTCAACTCCCTATGCTCAGATGTGGAGATATCT
TCTCCAGCCGAACCTCTTGGTCTTTTCCCATGCTCAGCACCACAGGCCCTCTCAGG
ACACACCCAGAGCTGCGGAAGAGCTAATGTCCTCAAGGGGTATCCAGCGCTGACTT
GCTGAGTTGGACTCAGGCAGGCAAGCCTGGTTTACCAGCCTTCTCAGTTAGCACAGAG
ACGCCAATTGGAACCTCGAAGACTTGTGTCAGAAATTTCTGGGCGGAACTTCTGG
GGTTGCTGTGAGCTTTTGGGCTTCTCAGCTTCAGATGCAAGTGAGTGGGTGTCTCAC
TGAGAAGCAAAGAGAAGAGAACAGGAGAAAGGTGTTTCCCTTGAAGTGCAGGAACTT
ATAAAGAAACTTAGCTTCTCTGGAGCAGTCAGCGTCAGAGTTCTGTCTTGACACCT
GAGTCTCTCCACAAGGCTGTGAAGAGGAAACCTTCTGGGGCTGGGTGCCATG

Red: ERE

Black: NF- κ B1 and NF- κ B2

Blue: SP1

Green: NF-AT

AP-1 (AP-1.1, AP-1.2, and AP-1.3), one SP1, one DRE, and two NF-AT motifs (Fig. 6A). Likewise, we identified the following transcription factor binding motifs in mouse FasL promoter: one ERE, two NF- κ B (NF- κ B1 and NF- κ B2), one SP1, and three NF-AT motifs (Fig. 6B).

DES-Induced Transcription Factor Bind with Various *cis*-Regulatory Motifs Present in Mouse Fas or FasL Promoters. Next, we performed *in vitro* studies to examine DES-induced transcription factors and their binding affinity with various *cis*-regulatory motifs present in the Fas or FasL promoters. As described under *Materials and Methods*, nuclear extracts prepared from T cells treated with VEH or DES (10 μ M/ml) were incubated together with various double-stranded probes (*cis*-regulatory motifs) of the Fas or FasL promoters as suggested by Clontech Laboratory, Inc.. The binding affinity was determined by performing chemiluminescent assays (Clontech Laboratory, Inc.) and analyzed by Victor² (PerkinElmer Life and Analytical Sciences). We observed significantly higher binding of ERE probes of both Fas and FasL with nuclear extracts isolated from DES-treated T cells compared with VEH-treated nuclear extracts (Fig. 7A). We did not observe a significant difference in the binding of the DRE probe with either of the nuclear extracts

Fig. 6. The *cis*-regulatory motifs present in Fas and FasL promoter. A, sequence of Fas promoter and various regulatory motifs present in the promoter. B, sequence of FasL promoter and various *cis*-regulatory motifs.

(Fig. 7A). These results demonstrated that DES may regulate Fas or FasL expression through ERE but not through DRE. In addition, we did not observe any DRE binding motif in the FasL promoter (Fig. 6B). Various NF- κ B probes of Fas and FasL showed different binding affinity with nuclear extracts from DES- or VEH-treated T cells. NF- κ B2 and NF- κ B3 probes of the Fas promoter showed strong binding affinity, whereas NF- κ B1, NF- κ B4, and NF- κ B5 showed no or low binding affinity with nuclear extracts isolated from DES-treated T cells compared with VEH-treated T cells (Fig. 7B). Likewise, the NF- κ B2 probe of FasL showed significant binding affinity, whereas the NF- κ B1 probe of FasL showed low or no binding affinity with DES-treated nuclear extracts compared with VEH-treated nuclear extracts (Fig. 7B). The NF-AT2 probe of Fas and NF-AT1 and NF-AT2 probes of FasL showed significant binding affinity with DES-treated nuclear extract compared with VEH-treated nuclear extract (Fig. 7C). We did not observe significant binding differences in the NF-AT1 probe with DES- or VEH-treated nuclear extracts (Fig. 7C). Of the three AP-1 probes of Fas promoter, two (AP-1.1 and AP-1.2) showed moderate binding affinity with DES-treated nuclear extract compared with VEH-treated nuclear extract (Fig. 7C). The results obtained from these studies demonstrated that various motifs of Fas or FasL

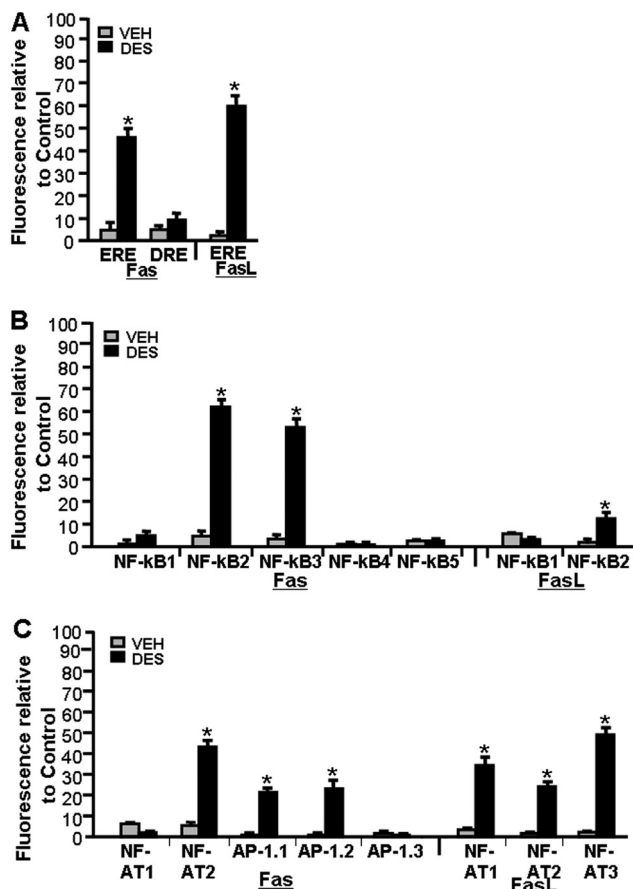


Fig. 7. Identification of binding affinity of *cis*-regulatory motifs present on the Fas and FasL promoters with DES-generated transcription factors in T cells. **A**, binding affinity of ERE and DRE motifs of the Fas promoter and ERE motif of the FasL promoter with transcription factors generated by DES or VEH in T cells. The ERE motif of both Fas and FasL promoters showed significantly higher binding affinity with transcription factors harvested from DES-treated T cells compared with transcription factors from VEH-treated T cells. The DRE motif of the Fas promoter showed low and insignificant binding affinity with transcription factors from DES-treated T cells compared with VEH-treated T cells. **B**, binding affinity of five NF-κB (NF-κB1, NF-κB2, NF-κB3, NF-κB4, and NF-κB5) motifs of the Fas promoter and two NF-κB (NF-κB1 and NF-κB2) motifs of the FasL promoter with transcription factors from DES- or VEH-treated T cells. The NF-κB2 and NF-κB3 motifs of the Fas promoter showed significantly higher binding affinity with transcription generated from DES-treated T cells compared with VEH-treated T cells. The NF-κB1, NF-κB4, and NF-κB5 motifs of the Fas promoter showed very low and insignificant binding affinity with transcription factors from either DES- or VEH-treated T cells. The NF-κB2 motif of the FasL promoter showed moderate, but significant, binding affinity with transcription factors from DES-treated T cells compared with VEH-treated T cells. The NF-κB1 motif of the FasL promoter showed very low and insignificant binding affinity with transcription factors from both DES- and VEH-treated T cells. **C**, binding affinity of NF-AT (NF-AT1 and NF-AT2 motifs of the Fas promoter and NF-AT1, NF-AT2, and NF-AT3 motifs of the FasL promoter) and AP-1 (AP-1.1, AP-1.2, and AP-1.3) motifs of the Fas promoter. NF-AT2 of the Fas promoter and all three NF-AT motifs of the FasL promoter showed significantly higher binding affinity with transcription factors from DES-treated T cells compared with VEH-treated T cells. Likewise, the AP-1.1 and AP-1.2 motifs of the Fas promoter showed significant binding affinity with transcription factors from DES-treated T cells compared with VEH-treated T cells. The vertical bars represent mean \pm S.E.M. from three independent experiments. *, significant ($p < 0.05$) difference in binding affinity of various motifs of Fas or FasL promoters with transcription factors from DES- and VEH-treated T cells.

promoter showed variable binding affinity with nuclear extract isolated from DES-treated T cells, demonstrating their role in the DES-mediated regulation of Fas or FasL expression.

DES-Induced Transcription Factors Bind with ERE and NF-κB *cis*-Regulatory Motifs Present in Fas or FasL Promoter. To further confirm the participation of various *cis*-regulatory motifs present in Fas or FasL promoters, we selected ERE, NF-κB1, and NF-κB2 motifs to verify their participation in DES-mediated regulation of Fas and FasL expression. The selection of ERE and NF-κB (NF-κB1 and NF-κB2) motifs of Fas and FasL was based on their binding affinity with DES-induced transcription factors (Fig. 7, A and B) that we obtained after chemiluminescent assays. ERE and NF-κB2 motifs of both Fas and FasL demonstrated high binding affinity, whereas NF-κB1 showed no or very low binding affinity with DES-induced transcription factors (Fig. 7, A and B). We performed EMSAs using radiolabeled single-strand loop ERE, NF-κB1, and NF-κB2 probes of Fas or FasL incubated together with NEs from vehicle- or DES-treated T cells. We observed shifts in the mobility of Fas and FasL ERE probes in the presence of nuclear extracts from T cells treated with DES (Fig. 8A, left for Fas ERE and right for FasL ERE). We also observed a shift in NF-κB2 probes of both Fas (Fig. 8B, left) and FasL (Fig. 8B, right) in the presence of NE from DES-treated T cells. No mobility shift of Fas and FasL ERE and NF-κB2 probes was observed when they were incubated without nuclear extract or incubated with VEH-treated nuclear extracts (Fig. 8, A and B). Furthermore, mobility shift of Fas and FasL ERE and NF-κB2 probes in the presence of nuclear extract was reversed when cold (unlabeled DNA) ERE or NF-κB2 probes were added in the reaction mixture (Fig. 8, A and B). In addition, when mutant ERE or NF-κB2 probes were used, there was no shift in probes (Fig. 8, A and B). However, when NF-κB1 probes of Fas or FasL were used, there was no mobility shift (Fig. 8C). The data obtained from these studies confirmed that DES induced transcription factors bound with ERE and NF-κB2 motifs of Fas and FasL promoters, demonstrating their role in DES-mediated regulation of Fas or FasL expression.

Discussion

Exposure to DES has been shown to increase the risk for breast cancer in DES mothers and a life time risk of cervicovaginal cancers in DES daughters (Giusti et al., 1995). It has also been linked to a wide range of abnormalities, including immune system disorders such as increased incidence of autoimmunity, cancer, and certain infections in DES offspring (son or daughter) (Giusti et al., 1995). Such studies support the fetal basis of adult disease, which states that exposure of fetus to nutritional, environmental, and other forms of stress can affect the health at later stages of life (Barker et al., 2002; Phillips, 2006). Previous studies from our laboratory have shown DES induces thymic atrophy and decreased thymic cellularity in mice (Brown et al., 2006a,b). Frawley et al. (2011) have recently reported gene expression alterations in immune system pathways in the thymus after exposure to immunosuppressive chemicals. In this study, we investigated DES-mediated thymic atrophy in neonatal mice and the regulation of Fas and FasL expression and examined the mechanisms of transcriptional regulation of Fas and FasL genes. To this end, we first examined the effects of DES in the thymic of mother and neonatal mice by performing *in vivo* experiments. Next, we determined the expression of Fas and FasL in fetal thymic cells and T cells in the presence or absence of

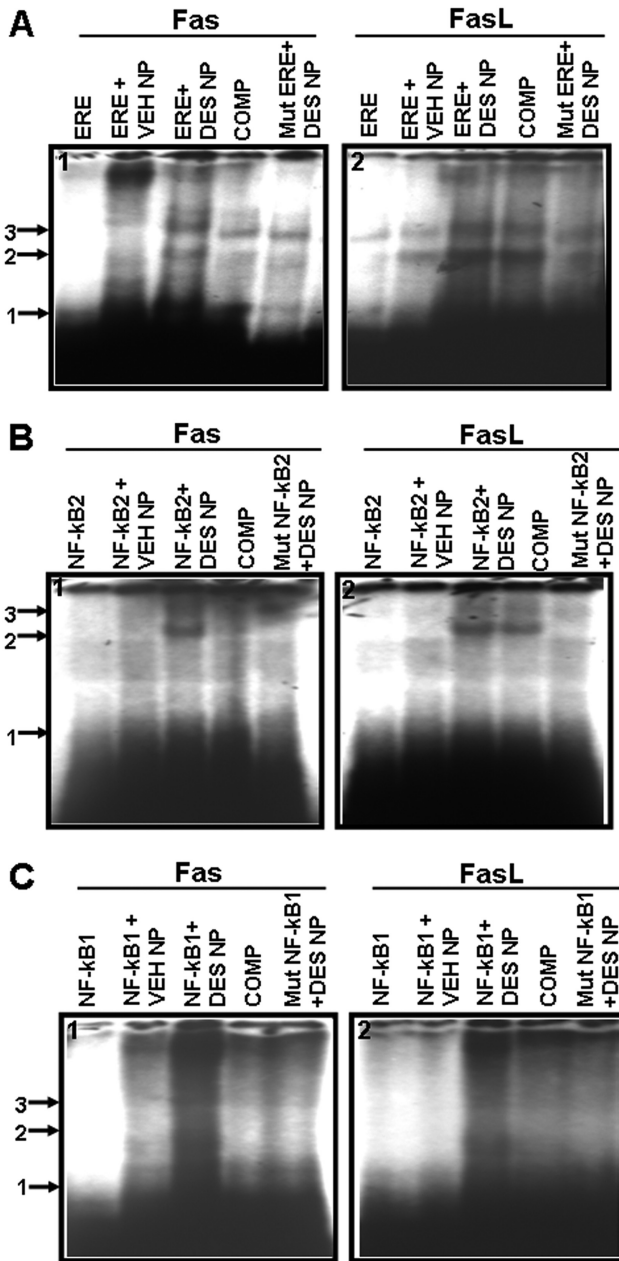


Fig. 8. EMSA analysis of ERE, NF- κ B1, and NF- κ B2 motifs of Fas and FasL promoters. **A**, single-strand hairpin loop probes containing the ERE motif of mouse Fas and FasL promoters were generated. Nuclear extract was generated from DES-treated T cells, and 4–5 μ g of nuclear extract was used in each reaction. Radiolabeled (P^{32}) ERE probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant ERE probe. Left, arrow 1 shows the Fas ERE probe DNA band, and arrows 2 and 3 show ERE-NE complexes. The lanes are labeled as ERE (ERE probe without NE), ERE+VEH NE (ERE probe + VEH-treated NE), ERE+DES NE (ERE probe + DES-treated NE), COMP [ERE probe + DES NE + cold (unlabeled) ERE probe for competition], and Mut ERE+DES NE (mutant ERE probe + DES-treated NE). Right, similar lane labeling but mouse FasL ERE probes have been used. **B**, single-strand hairpin loop probes containing the NF- κ B2 motif of Fas and FasL promoters were generated. Radiolabeled (P^{32}) NF- κ B2 probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant NF- κ B2 probe. Left, arrow 1 shows Fas NF- κ B2 probe DNA band, and arrows 2 and 3 show NF- κ B2-NE complexes. The lanes are labeled as NF- κ B2 (NF- κ B2 probe without NE), NF- κ B2 + VEH NE (NF- κ B2 probe + VEH-treated NE), NF- κ B2 + DES NE (NF- κ B2 probe + DES-treated NE), COMP [NF- κ B2 probe + DES NE + cold (unlabeled) NF- κ B2 probe for competition], and Mut NF- κ B2 + DES NE (mutant NF- κ B2 probe + DES-treated NE). Right, similar lane labeling but mouse FasL NF- κ B2 probes have been used. **C**, single-strand hairpin loop probes containing various NF- κ B1 motifs of Fas and FasL promoters were generated. Radiolabeled (P^{32}) NF- κ B1 probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant NF- κ B1 probe. Left, arrow 1 shows Fas NF- κ B1 probe DNA band, and arrows 2 and 3 show NF- κ B1-NE complexes. The lanes are labeled as NF- κ B1 (NF- κ B1 probe without NE), NF- κ B1 + VEH NE (NF- κ B1 probe + VEH-treated NE), NF- κ B1 + DES NE (NF- κ B1 probe + DES-treated NE), COMP [NF- κ B1 probe + DES NE + cold (unlabeled) NF- κ B1 probe for competition], and Mut NF- κ B1 + DES NE (mutant NF- κ B1 probe + DES-treated NE). Right, similar lane labeling but mouse FasL NF- κ B1 probes have been used.

DES and investigated the role of Fas and FasL promoters in DES-mediated regulation. We also examined the participation of *cis*-regulatory motifs in DES-mediated regulation of Fas and FasL expression.

The results obtained from the current study showed that DES induces thymic atrophy and significant changes in thymic cellularity of both mothers (Fig. 1A) and fetuses (Fig. 1B). In addition, there was significant apoptosis in thymic cells of both mother and fetuses after DES exposure compared with VEH (Fig. 2). Upon analysis of Fas and FasL expression in fetal thymic cells, there was a significant increase in Fas and FasL expression exposed to DES compared with fetal thymic cells exposed to VEH (Fig. 3, A and B). Furthermore, expression of Fas and FasL in unactivated and activated T cells in the presence of DES was significantly up-regulated in comparison with VEH (Fig. 4). In addition, DES regulated Fas or FasL expression via the ER because there was complete blockade of Fas or FasL expression in the presence of TAM, an antagonist of ER (Fig. 4). The data from the present study demonstrate that DES adversely affects fetal thymus by causing changes in thymic cellularity, up-regulating Fas and FasL expression, and causing apoptosis in thymic cells.

Previous studies from our laboratory have demonstrated that DES altered T-cell differentiation in the thymus by interfering with positive and negative selection processes, which in turn modulated the T-cell repertoire in the periphery (Brown et al., 2006a). In another study, Nair and Shaha (2003) reported DES-induced apoptosis in spermatogenic cells through the increased expression of Fas and FasL in rats. They have also shown that DES-mediated apoptosis did not occur in Fas-deficient *lpr* mice (Nair and Shaha, 2003). Together, these studies suggest that exposure to DES during development severely affects the thymus of neonatal mice and may have a long-lasting impact on the immune functions, leading to various health-associated problems and ailments.

The mechanisms through which DES up-regulates Fas and FasL expression in immune cells is unclear. The current study demonstrated that DES regulates Fas and FasL expression through their promoters as evidenced from the data we generated using luciferase assays in the absence or presence of DES. There was increased luciferase expression in both DES-treated EL4 cells and cells that were transfected with constructs containing full-length Fas or FasL promoter (Fig. 5). In an earlier study, we noted that aryl hydrocarbon receptor activation by TCDD up-regulated of Fas and FasL expression through promoters (Singh et al., 2007). Thus, it is likely that ER activation by DES also causes similar effects and regulates Fas and FasL expression through their promoters.

The regulation of a gene depends on *cis*-regulatory motifs

NF- κ B2 probe + DES-treated NE). Right, similar lane labeling but mouse FasL NF- κ B2 probes have been used. **C**, single-strand hairpin loop probes containing various NF- κ B1 motifs of Fas and FasL promoters were generated. Radiolabeled (P^{32}) NF- κ B1 probes were either directly used or used after incubation with NE or NE containing cold DNA (unlabeled, COMP) or NE containing mutant NF- κ B1 probe. Left, arrow 1 shows Fas NF- κ B1 probe DNA band, and arrows 2 and 3 show NF- κ B1-NE complexes. The lanes are labeled as NF- κ B1 (NF- κ B1 probe without NE), NF- κ B1 + VEH NE (NF- κ B1 probe + VEH-treated NE), NF- κ B1 + DES NE (NF- κ B1 probe + DES-treated NE), COMP [NF- κ B1 probe + DES NE + cold (unlabeled) NF- κ B1 probe for competition], and Mut NF- κ B1 + DES NE (mutant NF- κ B1 probe + DES-treated NE). Right, similar lane labeling but mouse FasL NF- κ B1 probes have been used.

present in its promoter. The number of *cis*-regulatory motifs present in a promoter varies from gene to gene. In addition, the participation of a *cis*-regulatory motif depends on the transcription factors generated by various mechanisms. To understand DES-mediated regulation of Fas and FasL expression, we performed sets of experiments to determine the role of their promoters and the *cis*-regulatory motifs present in the promoters. To this end, we first identified various *cis*-regulatory motifs present in the Fas or FasL promoters as described under *Materials and Methods*. The data obtained from the analysis showed the presence of several *cis*-regulatory motifs in both Fas (Fig. 6A) and FasL (Fig. 6B) promoters. Upon analysis of binding affinity of these *cis*-regulatory motifs of Fas or FasL promoter with DES-generated transcription factors, the results showed participation of several *cis*-regulatory motifs of Fas or FasL promoter in their regulation (Fig. 7). The important *cis*-regulatory motifs of Fas promoter that showed binding affinity with DES-generated transcription factors from T cells were ERE, NF-AT2, AP-1.1, AP-1.2, NF- κ B2, and NF- κ B3 (Fig. 7). *cis*-regulatory motifs of FasL promoter that showed binding affinity with DES-generated transcription factors were ERE, NF-AT1, NF-AT2, and NF- κ B2 (Fig. 7). The DRE motif of the Fas promoter showed no or minimal binding affinity with DES-generated transcription factors (Fig. 7A). The binding of DES-induced transcription factors with various *cis*-regulatory motifs of Fas or FasL promoter was further confirmed by performing EMSAs. The shift in probe mobility obtained from EMSAs confirmed the binding of ERE and NF- κ B2 motifs of both Fas and FasL with DES-generated transcription factors (Fig. 8, A and B). In addition, the shift in mobility of the probes was specific because cold probes (nonradioactive) reduced the shift in mobility of the probes (Fig. 8, A and B). Moreover, the mutant probes of ERE or NF- κ B2 did not show mobility shift of the probes when incubated together with DES-generated transcription factors (Fig. 8, A and B). Furthermore, NF- κ B1 probes of Fas or FasL did not show mobility shift in the presence of DES-generated transcription factors (Fig. 8C). All of these results demonstrated that DES caused up-regulation of Fas or FasL expression by generating transcription factors and binding with *cis*-regulatory motifs present in Fas or FasL promoter regulated their expression.

Payton-Stewart et al. (2010) have shown ER-mediated gene expression through ERE *cis*-regulatory motif present in the promoter. They have demonstrated that glyceollin I enantiomers that bind with ER regulate several genes through the ERE of their promoter. Frasier et al. (2009), on the other hand, have shown extensive positive cross-talk between the ER and NF- κ B and that these factors may act together to regulate several genes. In another study, Guzeloglu-Kayisli et al. (2008) reported that interaction between the ER and NF- κ B may affect the regulation of responsive genes by down-regulating NF- κ B-dependent gene activation and directly preventing DNA binding. Safe and Kim (2008) have shown ER-mediated regulation of genes through the AP-1 *cis*-regulatory motif. All of these studies demonstrate the role of ER, various transcription factors, and associated *cis*-regulatory motifs involved in the induction of various genes.

The induction and expression of Fas and FasL on cells could constitute a double-edged sword. Underexpression or mutation in Fas or FasL can cause various disorders, includ-

ing autoimmune lymphoproliferative syndrome and susceptibility to lymphoid cancers (Kovacs et al., 1997b; Grønbaek et al., 1998; Straus et al., 1999). In contrast, overexpression of Fas or FasL results in uncontrolled apoptosis, leading to cardioretinopathology, neurodegenerative disorders, and failure to clear pathogens (Kovacs et al., 1997a; Sharma et al., 2000). The continuous FasL expression in testes has been shown to cause autoimmunity, leading to fertility problems (Hu et al., 2003). whereas the immune privilege status of various organs (eyes and testes) is partially caused by the high expression of FasL in these organs (Griffith et al., 1995). FasL constitutively expressed in the thymus plays an important role in the ontogenesis and negative selection of T cells (Kabelitz, 1993), but up-regulated expression of FasL in thymus also leads to development of autoimmunity (Kobata et al., 1997; Brochu et al., 1999). In addition, in the thyroid gland, inflammation leading to interaction between Fas and FasL can cause thymocyte destruction and trigger Hashimoto's thyroiditis (Stassi et al., 2000). In the current study, we noted that Fas and FasL were up-regulated in the T cells in the presence of DES, which suggests potential deleterious effects on the development of the normal immune system.

In conclusion, the current study demonstrates that prenatal exposure to DES triggers significant alterations in apoptotic molecules expressed on immune cells that play a critical role in the regulation of autoimmunity and cancer. The mechanisms include DES-induced transcriptional regulation of Fas and FasL genes involving ER-mediated signaling and the participation of ERE and/or NF- κ B motifs present on their promoters. Furthermore, our studies suggest that such alterations in apoptotic molecules in the thymus may affect T-cell differentiation and have long-term consequences for the immune functions.

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Authorship Contributions

Participated in research design: N. P. Singh, U. P. Singh, P. S. Nagarkatti, and M. Nagarkatti.

Conducted experiments: N. P. Singh and U. P. Singh.

Contributed new reagents or analytic tools: N. P. Singh, P. S. Nagarkatti, and M. Nagarkatti.

Performed data analysis: N. P. Singh, U. P. Singh, P. S. Nagarkatti, and M. Nagarkatti.

Wrote or contributed to the writing of the manuscript: N. P. Singh, U. P. Singh, P. S. Nagarkatti, and M. Nagarkatti.

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